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Carbohydrate and Ethane Release with *Erwinia carotovora* Subspecies *betavasculorum*–Induced Necrosis

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Abstract *Erwinia carotovora* subspecies *betavasculorum*, also known as *E. betavasculorum* and *Pectobacterium betavasculorum*, is a soil bacterium that has the capacity to cause root rot necrosis of sugarbeets. The qualitatively different pathogenicity exhibited by the virulent *E. carotovora* strain and two avirulent strains, a *Citrobacter* sp. and an *Enterobacter cloacae*, was examined using digital analysis of photographic evidence of necrosis as well as for carbohydrate, ethane, and ethylene release compared with uninoculated potato tuber slices. Visual scoring of necrosis was superior to digital analysis of photographs. The release of carbohydrates and ethane from potato tuber slices inoculated with the soft rot necrosis-causing *Erwinia* was significantly greater than that of potato tuber slices that had not been inoculated or that had been inoculated with the nonpathogenic *E. cloacae* and *Citrobacter* sp. strains. Interestingly, ethylene production from potato slices left uninoculated or inoculated with the nonpathogenic *Citrobacter* strain was 5- to 10-fold higher than with potato slices inoculated with the pathogenic *Erwinia* strain. These findings suggest that (1) carbohydrate release might be a useful measure of the degree of pathogenesis, or relative virulence; and that (2) bacterial suppression of ethylene formation may be a critical step in root rot disease formation.

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Introduction

Many bacterial species within the genus *Erwinia* (proposed *Pectobacterium*) of the *γ-Proteobacteria* cause soft rot diseases on plants; and while a number of virulence factors affect the phytopathogenic ability of these bacteria [19], it has long been realized that the tissue-macerating activity or "soft rot" characteristic of the disease is due to the activity of cell-wall-degrading enzymes that are produced and released by these plant pathogens [11]. The *Erwinia* are "brute-force" pathogens that produce large amounts of enzymes that disrupt the plant's cell wall [20], and it is the damage caused by these enzymes that is primarily responsible for the damage these pathogens cause to crops [18]. These secretions include a range of cellulolytic enzymes that attack the main constituent of the plant's cell walls as well as pectinolytic enzymes that attack the polysaccharide that cements the plant's cell wall components. The enzymes include cellulase, polygalacturonase, pectin lyase, pectate lyase, pectin methyl esterase, protease, and phospholipase [17]. Not only are there different classes of enzymes involved, but also each enzymatic activity may exist in multiple forms as isozymes. Perombelon [18], in a recent review article, concluded that pectinases are the most important virulence factors of the *Erwinia.* This fits with the conclusion by Hammerschmidt [9] that the most important step in the tissue maceration process involves the degradation of the pectic compounds that make up the middle lamella and primary cell wall.

A number of methods have been used to measure virulence. Ethylene, a plant hormone, is often formed by plants in response to a pathogen attack, but its formation may not correlate well with the degree of tissue damage [5, 13, 21]. Stronger correlations [13] have been

observed with the formation of ethane, which is released from damaged cell membranes and the resulting oxidation of linoleic and linolenic acids when tissue compartmentalization is disrupted by cell injury [7, 14]. Kimmerer and Kozlowski [13] found that there was a linear correlation between the release of ethane and tissue necrosis. In addition, they found that acetaldehyde and ethanol were also produced by some plants in response to tissue damage, but the production of these compounds was not as reliable a predictor of plant stress as was ethane production. Their recommendation was that all four compounds (ethylene, ethane, acetaldehyde, and ethanol) be measured.

The development of this present study began in conjunction with characterization studies of the phytopathogenic bacteria *E. carotovora* subspecies *betavasculorum* and our need to screen for strains of this organism that exhibited different levels of virulence. The time required for growing host plants, subsequent inoculation studies, and the development of disease symptoms was a major limiting factor in these studies, so we decided to develop a suitable rapid bioassay for virulence that would facilitate further studies of a possible link between different virulence factors in bacteria that cause soft rot diseases on tuber crops. The methods of analysis performed on infected and noninfected potato slices included ethylene and ethane formation, the release of soluble carbohydrates by damaged tissues, and the use of digital analysis of photographic images using two types of software.

Materials and Methods

Microorganisms

The proposal to rename the soft rot *Erwinia* as *Pectobacterium* [10] has not at this time been widely accepted, but the subsequent proposal [8] to elevate three subspecies of *Pectobacterium carotovora* to species rank is accepted. Therefore, in this paper we refer to the bacterial strain under study as *Erwinia betavasculorum*. This particular soil bacterium, also known as *Pectobacterium betavasculorum* [8], has the unique capacity to cause root rot necrosis of sugarbeets. The strain used in this study, a virulent derivative of the type strain CFBP2122, was isolated from sugarbeet roots that had been inoculated with the type strain from Gardan's laboratory in France. The identity of the virulent isolate was confirmed by partial 16S rDNA sequencing (MIDI Labs). The *Citrobacter* sp. and the *Enterobacter cloacae* strains were also isolated from the rhizosphere of greenhouse-grown sugarbeets, and were identified by partial 16S rDNA sequencing.

Potato Slice Inoculation

Russet potatoes were surface-sterilized as follows: two washes for 20 min in 15% (vol/vol) of a commercial 5.25% sodium hypochlorite and 0.01% sodium dodecyl sulfate, followed by several rinses with sterile water [15]. For disease progression/necrosis monitoring and photographic documentation, slices of potato several millimeters thick were cut and placed on agar plates containing Murashige and Skoog tissue culture medium [16] with Gamborg's vitamins and 5% phytogel (Sigma).

Fresh Russett potatoes were surface-treated as described above, aseptically sliced, and inoculated with 10 µL of a fresh overnight bacterial culture in Luria Bertani broth [2] containing a total of 10^7 colony-forming units spotted in the center of the potato slices (5 replications), which was then incubated at 28°C. At intervals, the potato slices were examined for necrosis and photographed. The degree of necrosis induced by the different *E. betavasculorum* strains was estimated using two methods. Both methods relied on the use of digital photographs taken from the same distance and taken under the same lighting conditions and coupled with an analysis of the image using either the Adobe Photoshop v. 5.02 or Adobe Photoshop Elements v. 4.0 software programs (Adobe Systems Inc.). The first method measured the darkening of the potato slices observed with infective strains. For this procedure, the edges of the potato slices were manually traced using the "lasso tool" and the discoloration of the slice was measured using the luminosity feature of the software. For the second method, the size of the necrosis was estimated by tracing the edges of the necrosis zone with the software's "lasso tool" and using the pixel counting option to provide a relative measurement of damaged tissue.

Carbohydrate, Ethane, and Ethylene Release studies

Russet potatoes, surface-treated as described above, were sampled using a 12-mm-diameter cork borer that had been placed into 70% isopropanol for 30 min, drained, and air-dried. "Cores" were trimmed to a length of 2.5 cm with a surface-sterilized razor blade. For gas sampling, cores were placed into sterile 10-mL serum vials and inoculated with 100 µL of overnight cultures or TY medium [1] only for the uninoculated control (7 replications for each treatment). Serum bottles were capped with sterile Teflon-coated septa and the vials were rolled on the countertop to distribute the culture over the potato core. An 18-gauge syringe needle was inserted into each septum for ventilation and the serum bottles were incubated in the dark at 28°C for 24 hr. After this initial incubation, the bottles were sealed (by removing the 18-gauge syringe needle) and incubated an additional

2.5 hr. After this second incubation, 2 mL of each bottle's atmosphere was analyzed by gas chromatograph to determine ethylene and ethane accumulation.

Gas Chromatography

A packed column gas chromatograph was used to measure ethylene and ethane. The instrument was equipped with a 2-mL inlet sample loop that was heated to 70°C. Carrier was helium at 30 mL min^{-1}. Chromatographic separation was performed using a 3.2-mm × 3-m Porapak N column (Supelco) heated to 115°C. Detection was by a flame ionization detector operated at 200°C.

Carbohydrate Release Assay

For carbohydrate release, surface-sterilized Russet potatoes cores were placed aseptically into autoclaved 15-mL test tubes and inoculated with 100 µL of 18hr-old cultures or TY medium only (as an uninoculated control). Each treatment was replicated three times. Tubes were capped with plastic test tube caps and rolled to distribute culture media over the potato core. Tubes were incubated in the dark at 28°C for 20 hr.

After the incubation, carbohydrates released by the action of pectic and cellulolytic enzymes were assayed. For this assay, 10 mL of deionized water was added to each test tube and the tubes were shaken on an orbital incubator for 15 min at 28°C and 100 rpm. Samples of the liquid were taken from each tube, centrifuged for 10 min at 15,300*g* at 4°C, and 0.5 mL of each supernatant was assayed for carbohydrate content using the phenol-sulfuric acid assay [4] as follows. To each 0.5-mL sample add 0.5 mL of a 5% (wt/vol) phenol solution and 2 mL of concentrated H_2SO_4. The H_2SO_4 must be added quickly. Tubes were then vortexed for 5 seconds, a marble was placed on the top, and the tubes were incubated for 30 min at 80°C and 100 rpm in a shaking water bath. After the incubation, the tubes were cooled and their absorbance at 490 nm was determined. Values were compared to a 0 to 300 ppm glucose standard curve.

Statistical Comparisons

Statistical evaluations given in the text and in figures are means ± standard error of the mean calculations made using the Instat® computer program's analysis of variance (ANOVA) and Tukey-Kramer Multiple Comparisons Test (GraphPad Software Inc.).

Results and Discussion

Digital Photographic Analysis of Infection

The inoculation of potato slices with the virulent *E. betavasculorum* strain, which has the capacity to cause root rot necrosis of sugarbeets, produced necrosis that was visually different from that exhibited by a the *Citrobacter* sp. [6] and *E. cloacae* strains isolated from the sugarbeet rhizosphere. The pathogenic *E. betavasculorum* formed areas of necrosis evident by tissue discoloration from white to brownish black and then dissolution in 2 to 3 days, whereas the *Citrobacter* sp. produced a slight brownish discoloration and the *E. cloacae* strain produced no discoloration (Fig. 1).

Two digital analysis methods were evaluated as methods for quantifying the extent of tissue necrosis. The first method used the image processing software's luminosity feature to measure the overall tissue discoloration of the whole surface of the potato. Despite a clear visual discernment of a treatment effect, this approach with the luminosity feature failed to detect a treatment effect; a one-way ANOVA of the treatments yielded a p value of 0.3070, considered not significant. A second approach using the luminosity feature to measure the luminosity or changes in the brightness of just the infected area gave much better

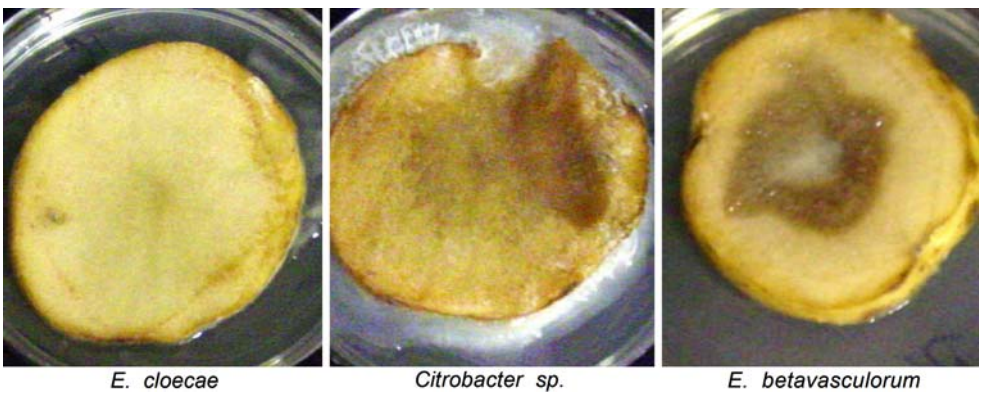

Fig. 1 Tissue necrosis induced by *E. cloacae, Citrobacter* sp., and *E. betavasculorum* after 3 days' incubation

results (Fig. 2) with a large and significant ($p < 0.001$) decrease in luminosity being observed with the virulent *E. betavasculorum* strain. The procedure was able to distinguish the virulent *E. betavasculorum* strain from the avirulent strains. However, the *Citrobacter* sp. also caused some darkening of the potato surface (Fig. 1), which shows up as a decrease in luminosity. This assay likely overestimated the virulence of the *Citrobacter* sp. strain. Nonetheless, the approach does provide a method for quantifying tissue discoloration.

A second image analysis method tested involved estimating the size of the area of necrosis by using the pixel counting feature of the software after using the software's lasso feature to manually mark the boundary necrosis zone. Results obtained with this method were variable, depending upon the ability of the user to estimate necrosis. The tendency was to overestimate the tissue damage. Results with this method indicated that the average size of the necrosis was numerically larger with the virulent *E. betavasculorum* strain (Table 1), but the method failed to measure a difference in infectivity of the virulent *E. betavasculorum* strain and the avirulent *Citrobacter* sp. strain. A *t*-test of the *E. betavasculorum* and *Citrobacter* sp. treatment data show that the two-tailed *p* value was 0.1839, considered not significant. The weakness of this procedure is that it is somewhat subjective in that it is dependent upon the user of the software to estimate the boundary of the necrosis zone and the degree of necrosis. Thus, the success of the procedure is dependent upon the operator. It may work well with some operators but not with others. The procedure did work very well as a means of measuring the size of an irregularly shaped area.

Table 1 Necrosis zone size estimated using the pixel counting feature available on commercial photographic software. Values are mean ± standard error of the mean, n = 5

Treatment	Necrosis zone area (mm^2)
E. cloeace	0 ± 0
Citrobacter sp.	368 ± 229
E. betavasculorum	723 ± 71

Carbohydrate Assay

We reasoned that carbohydrate release from wounded or infected tissue would occur as a result of the action of cell-wall degrading pectinolytic and cellulytic enzymes [11, 17, 18, 20]. Accordingly, we developed a simple carbohydrate release assay and tested it using plugs of potato inoculated with the three strains (Fig. 3). In this assay, about 5 ppm of glucose equivalents of soluble carbohydrate were released by uninoculated potato plugs, while the amount of

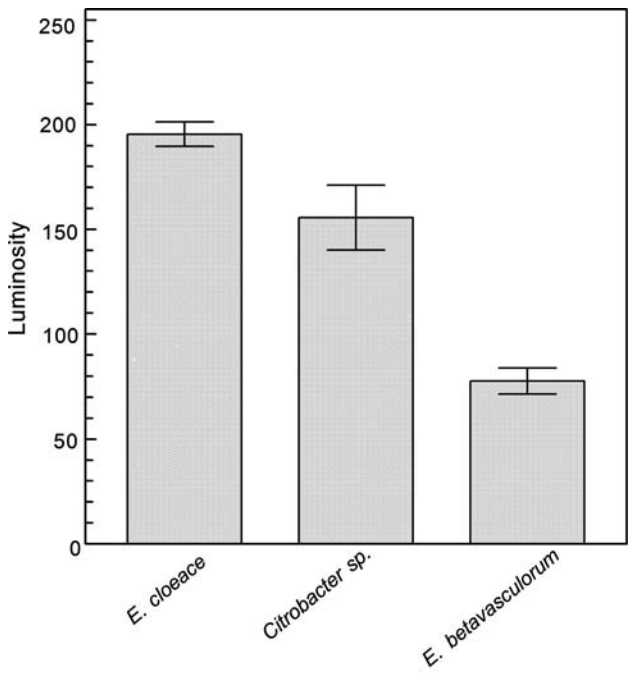

Fig. 2 Infected zone luminosity of potato slices inoculated with bacteria differing in virulence. Photographic luminosity is a measure, on a scale of 0–255, of the brightness of the object or area being photographed. The higher the number, the brighter the object or area. Infected tissues have a decreased luminosity

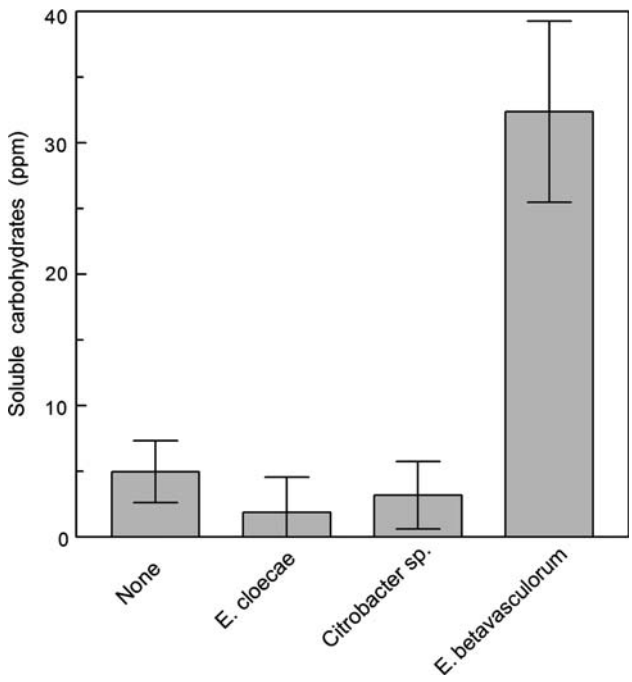

Fig. 3 Carbohydrate release by plugs from potatoes inoculated and incubated for 24 hr with bacteria that differ in virulence. Values are mean ± standard deviation, n = 3

carbohydrates released by potato plugs inoculated with the virulent *E. betavasculorum* strain were about 6-fold higher. Potato plugs inoculated with the avirulent strains had less sugar released than did the uninoculated controls and about one-tenth the level of sugar release caused by the pathogenic strain *E. betavasculorum*. As expected, an analysis of variance showed that the results with *E. betavasculorum* were significant. *E. betavasculorum* produced significantly more ($p > 0.05$) soluble carbohydrate than did the control, *E. cloacae*, or *Citrobacter* sp. treatments.

Ethane and Ethylene Assay

Ethane may also be produced by diseased or wounded plants and its production can be followed using gas chromatography. Ethane is thought to be more of an indicator of cell or tissue death than of stress [12, 13]. Therefore, we evaluated the formation of ethane by potato plugs as a measure of virulence by the three strains, and just as carbohydrate release was correlated with pathogenicity above, a similar and parallel positive correlation was also observed between the virulence of the strains used as an inoculum and ethane production from potato plugs after a 24-hr incubation (Fig. 4). Ethane accumulated to 0.85 ± 0.16 ppm in the atmosphere over potato plugs inoculated with the virulent *E. betavasculorum* strain. This is ~15 times greater than the accumulations that were observed with the avirulent *Citrobacter* sp. and *E. cloacae* strains and ~6 times higher than that observed with the uninoculated control. As expected, an analysis of variance shows that the results with *E. betavasculorum* were highly significant. *E. betavasculorum* produced significantly more ($p > 0.05$) ethane than did the control or *Citrobacter* sp. and *E. cloacae* treatments, whereas the *Citrobacter* sp. and *E. cloacae* treatments did not differ significantly from the control ($p < 0.05$).

Ethylene decreased by about 75% below the values produced by healthy tissues when the virulent strain was the inoculum and increased by about 1.5–2-fold when the avirulent strains were present (Fig. 4). These changes were not as large as was observed with ethane or with the release of carbohydrate. Ethylene is generally expected to increase when plants are exposed to a pathogen [3]; however, as has been noted in earlier studies, this is not always the case and ethylene production by stressed tissues can decrease [13]. We did not investigate the reason for this decrease in ethylene formation but hypothesize that the decrease in ethylene formation observed with the pathogenic *Erwinia* strain may have been due to the pathogen's ability to effectively repress the plant's production of signaling factors involved in an effective defense response.

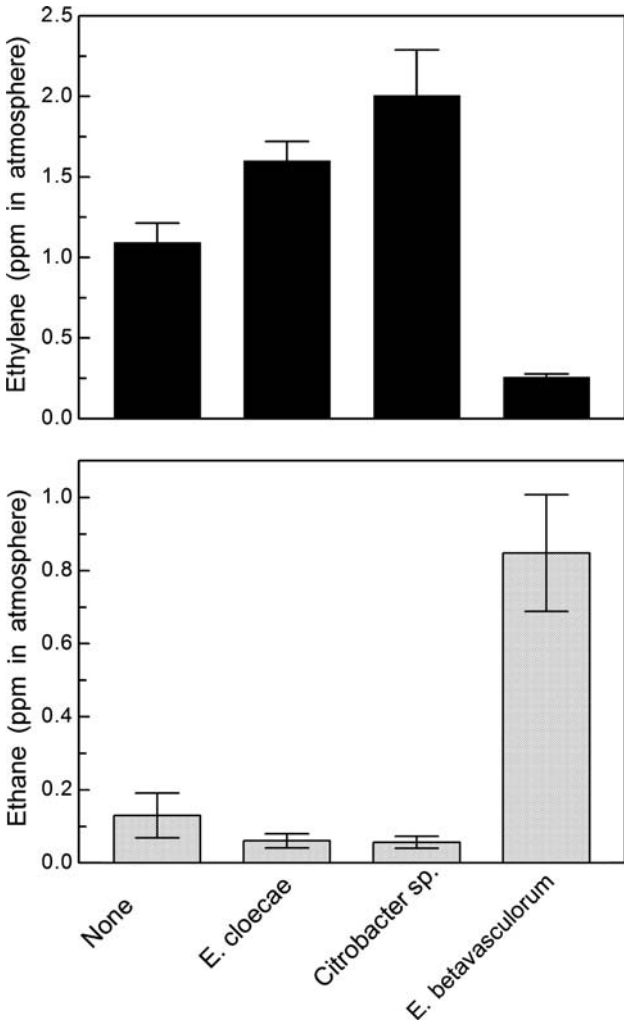

Fig. 4 Ethane and ethylene production by plugs from potatoes inoculated and incubated with bacteria that differ in virulence. Values are mean ± standard deviation, n = 7

Conclusions

Digital analysis of digital photographs of infected potato slices failed to produce a qualitative separation, or quantitative measure, of the pathogenicity of the bacterial species evaluated here. The first procedure, using luminosity to measure the darkening caused by a pathogenic *E. betavasculorum* strain, either underestimated the degree of necrosis or overestimated the degree of necrosis. Nonetheless, the procedure does provide a reproducible means of measuring tissue darkening. The second digital photographic attempt, using the pixel counting feature of the software to estimate the area of necrosis, yielded results that were numerically larger when pathogenic *Erwinia* was the inoculum but failed to yield a statistically valid increase over inoculation with the nonpathogenic *Citrobacter* sp. Better results, although somewhat subjective, can more readily be visually obtained by direct scoring of the extent of necrosis.

Both ethane production and carbohydrate release assays worked well and yielded a quantitative measure of virulence within a relatively short, ~24 hr, incubation time. Of these two assays the carbohydrate release assay was, in our opinion, the better of the two procedures, generally yielding more reliable results with fewer replicates. Although both spectrophotometers and gas chromatographs are common laboratory instruments, spectrophotometers are generally more common, and are usually less expensive to purchase and operate than gas chromatographs, and probably require less technical skill to operate. These data clearly establish the feasibility of monitoring the extent of soft rot necrosis by a simple colorimetric assay that monitors the release of soluble carbohydrates from potato plugs. Although potato was selected as test material for this study, other similar plant tuber materials should also work and may work better when matched with specific plant pathogens.

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